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## **Assessing whether disinfectants against the fungus *Batrachochytrium dendrobatidis* have negative effects on tadpoles and zooplankton**

Schmidt, B R ; Geiser, C ; Peyer, N ; Keller, N ; von Rütte, M

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**Assessing whether disinfectants against the fungus *Batrachochytrium dendrobatidis* have negative effects on tadpoles and zooplankton**

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**Abstract.** Chytridiomycosis is an emerging disease of amphibians that has led to global population declines and possible extinctions. Vectoring of the pathogen, *Batrachochytrium dendrobatidis* (*Bd*) by anthropogenic means is thought to be important in its spread. To limit further increase in the distribution of *Bd*, field biologists and amateur naturalists ought to disinfect their boots and materials. However, imprudent use of potentially harmful disinfectants may have unwanted negative side effects on amphibians. We used a factorial experiment to test whether commonly used disinfectants (bleach and Virkon S) affect tadpole performance and zooplankton abundance. At the high dose of bleach, all tadpoles and zooplankton died. Tadpole performance and zooplankton abundance in the low dose of bleach and Virkon S treatments were undistinguishable from the control. Therefore, when bleach is used as a disinfectant, it must not get in contact with amphibians. Virkon S appears to be a disinfectant that can be used against *Bd* with no detectable negative effects on tadpoles and zooplankton.

**Keywords:** chytridiomycosis, disinfectant, Virkon S, bleach, *Rana*, *Bufo*, tadpole

## Introduction

Emerging infectious diseases are on the rise and may represent a serious threat to biodiversity (Daszak et al., 2000; Jones et al., 2008). The emerging infectious disease chytridiomycosis is one particularly worrisome reason for the global decline of amphibians (Houlahan et al., 2000; 2001; Stuart et al., 2004). Chytridiomycosis, a disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), may be responsible for the extinction of many amphibian species (Stuart et al., 2004; Skerratt et al., 2007; Lips et al., 2008). *Bd* is being recorded in more and more localities on many continents and is likely to spread even further (Weldon et al., 2004; Garner et al., 2005; Ouellet et al., 2005; Lips et al., 2008, Woodhams et al., 2008). Chytridiomycosis was characterized in the IUCN Amphibian Conservation Action Plan “as the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction” and “there is growing consensus among scientists that the spread of chytridiomycosis has driven and will continue to drive amphibian species to extinction at a rate unprecedented in any taxonomic group in human history” (Gascon et al., 2007).

While it is possible to treat individual amphibians against the disease and to disinfect laboratory and field equipment (Johnson et al., 2003; Woodhams et al., 2003; Young et al., 2007; Pessier, 2008; Garner et al., 2009), there is no method yet available to treat amphibians against the disease in the wild or to eliminate the fungus in amphibian habitats (Gascon et al., 2007; Young et al., 2007). Because the fungus persists in habitats that it invaded (Retallick et al., 2004; Woodhams et al. 2008), amphibian conservationist and government authorities have argued that it is of paramount importance to prevent the further spread of *Bd* (NSW Parks and Wildlife Service, 2001; Australian Government Department of Environment and Heritage, 2006; Fisher and Garner, 2007; Gascon et al., 2007; Dejean et al., 2007; Young et al., 2007).

Some of the chemicals used to disinfect materials in order to stop the spread of *Bd* are known to be highly toxic (e.g., bleach; Racioppi et al., 1994). While advocating disinfecting boots, materials and other materials used by field biologists and amateur naturalists, we have often heard the concern that the widespread use of potentially harmful disinfectants may have negative effects on amphibians and other wetland animals (B. R. Schmidt, personal observation). For example, the uncautious use of

bleach might lead to the contamination of ponds and wetlands with a highly toxic chemical and may impair the growth of amphibians or lead to elevated mortality. We therefore decided to experimentally assess the effects of two commonly recommended and used disinfectants (bleach and Virkon S) against *Bd* on the performance of tadpoles.

## Material and methods

### *Experimental setup*

We conducted a mesocosm experiment to assess the potential harmful effects of disinfectants against *Bd* on tadpole performance and zooplankton abundance (Rowe and Dunson, 1994). We used green tubs (0.28 m<sup>2</sup>, 80 l) as mesocosms. Mesocosms were located outdoors on a fenced field on the Irchel campus of the University of Zurich, Switzerland. Tubs were filled with tap water. We added 40 g of dry leaves from a nearby forest to increase structural complexity and to serve as the basis for the mesocosm food web. In addition, we added aliquots of phyto- and zooplankton and an adult snail (*Lymnaea* sp.), all collected from nearby ponds. Tadpoles grow well in such experimental mesocosms (Van Buskirk, 2002).

We tested two commonly used disinfectants. Both bleach and Virkon S are known to be highly efficient disinfectants and kill *Batrachochytrium dendrobatidis* reliably (Johnson et al., 2003). The two disinfectants have the advantage that they can be used to disinfect materials, boots, etc. while doing field work. We used bleach at the concentration that is commercially available in local stores (2%). Virkon S was used at the concentration 10 g l<sup>-1</sup>.

Disinfectants were applied at two doses. In the high dose treatment, we attempted to mimic the amount of disinfectant that might run off a rubber boot. To quantify this amount, we immersed rubber boots repeatedly in water and measured the volume of water that dripped off the rubber boot. All the repetitions gave about 0.04 l per rubber boot pair as a result. The low dose treatment was a tenth of the high dose, i.e. 0.004 l. We added 0.06 l of tap water to the high dose and 0.096 l of tap water to the low dose. We added 0.1 l of tap water to the controls such that all treatments received the same volume of liquid.

The high dose represents a worst case scenario. No field biologist is likely to disinfect boots and walk directly into a pond (which would have much larger volumes than our 80 l mesocosms). However, if there are no effects of the disinfectants at such high doses, then one should not expect effects at much lower,

realistic doses of disinfectants that may get into a pond. Disinfectants or tap water (controls) were added once a week.

The two doses were crossed with two disinfectants which gave six treatment combinations. The two controls (high and low dose of tap water) were in fact identical. Treatment combinations were replicated five times. Treatment combinations were assigned randomly to tubs. In each replicate we added ten tadpoles of *Rana temporaria* and ten tadpoles of *Bufo bufo*. Although *Bufo bufo* is listed as vulnerable on the Swiss amphibian red list, it is still widespread and relatively common (Schmidt and Zumbach, 2005). *Rana temporaria* is listed as “least concern”. At the start of the experiment, *Rana temporaria* and *Bufo bufo* tadpoles had an average mass of 20.2 mg and 17.6 mg, respectively. We did not record developmental stages.

#### *Response variables and statistical analysis*

Response variables included behaviour, mass and survival of tadpoles (Bridges and Semlitsch, 2005). We counted daily (except some weekends) the number of tadpoles visible and counted how many of those were feeding. Observation time per experimental unit was one minute. The proportion feeding was used as the behavioural response to treatments because feeding is an ecologically important trait (Werner and Anholt, 1993; Rist et al., 1997). Feeding rate may affect growth rate and mass. Mass and survival have evident implications for population dynamics (Altwegg and Reyer, 2003; Schmidt et al., 2005). Mass and survival were measured after three weeks when the experiment was halted. In mesocosm experiments like ours, effects of toxicants on performance are usually apparent after three weeks (Relyea, 2003).

In addition to measuring tadpole performance traits, we quantified zooplankton abundance. To do so, we collected one liter of water on the day the experiment was halted. We then counted all daphnids, copepods and ostracods.

Statistical analyses were done using the program R (R Development Core Team, 2007). Survival and zooplankton abundance were analysed using general linear models with binomial and Poisson errors, respectively. The linear model included the factors disinfectant, dose and the interaction. Mass and the proportion of tadpoles feeding (after angular transformation; the proportion is the mean of all observations where tadpole behaviour was quantified) were analysed using a linear model with normal errors. Because all tadpoles died in the high dose of bleach treatment, the data could not be analysed as

described above. Instead, we analysed the data using a single explanatory factor with four treatment levels: control (both controls were pooled), low dose of bleach, low dose of Virkon and high dose of Virkon. For mass, the number of tadpoles surviving was used as a covariate.

Type I error probability was 5%. In the context of this experiment, this is an application of the precautionary principle because it gives more conservative results. That is, an effect of a disinfectant is more likely to be considered significant and one is more likely to diagnose a (probably negative) effect of disinfectants on tadpoles.

## Results

Tadpole behaviour (proportion of visible tadpoles that were feeding) was unaffected by the experimental treatments (figure 1); linear models showed no significant effects of treatments (*Rana temporaria*:  $F_{3,21}=1.02$ ,  $P=0.40$ ; *Bufo bufo*:  $F_{3,21}=1.21$ ,  $P=0.32$ ).

Survival varied among treatment combinations. All tadpoles, all zooplankton and some of the snails died in the high dose of bleach treatment. Our behavioural observations showed that all mortality occurred within one or two days after the first addition of bleach. Among the remaining treatment combinations, there was little variation (figure 2). Because all tadpoles in the high dose of bleach treatment died, the effects of disinfectant, dose and the interaction were highly significant for both species (table 1). We used a GLM with binomial errors to compare the control and the low dose of bleach treatment (figure 2). This GLM showed a significant difference for both *Rana* and *Bufo* ( $P=0.0013$  and  $P=0.0005$ , respectively).

Mass of *Bufo bufo* tadpoles was not affected by experimental treatments (figure 2) but it responded significantly to density (table 2). Mass of *Rana temporaria* tadpoles was significantly affected by both number of survivors (= density) and treatments (figure 2, table 2). This was primarily because tadpoles had higher masses in the low dose of bleach treatment ( $\bar{x}=0.52$  g vs.  $\sim 0.4$  g in the other treatments). It should be

noted that higher mortality occurred primarily in the low dose of bleach treatments. Hence, the effects of density and treatment are partially confounded.

Because there was 100% zooplankton mortality in the high dose of bleach treatment (figure 3), the effects of disinfectant, dose and the interaction were significant (disinfectant:  $P < 0.0001$ ; dose:  $P = 0.0400$ ; interaction:  $P < 0.0001$ ). Among the remaining treatment combinations there was no evident treatment effect (figure 3) and variation within treatment combinations was high.

## Discussion

The use of disinfectants to prevent the spread of *Bd* should not have negative effects on amphibians, especially in populations where the fungus is not yet present. Our experiment showed that bleach can be harmful to tadpoles and zooplankton whereas Virkon S neither affected tadpole behaviour, growth nor mortality (figure 1 and 2). Similarly, zooplankton was unaffected by Virkon S (figure 3).

Interestingly, tadpole mass was highest in the low bleach treatment. This was the case for all replicates, even those where no mortality occurred (figure 2, table 2). This may be a hormetic response to bleach, i.e. a stimulation effect at low doses of a toxicant that is harmful at high doses (Forbes, 2000). However, hormesis does not necessarily lead to higher fitness of individuals in this treatment (Forbes, 2000). A more speculative interpretation of the seemingly beneficial effect of a low dose of bleach might be that bleach perhaps killed tadpole parasite and harmful microorganisms.

The doses of bleach and Virkon S that we applied to experimental mesocosms were very high in comparison to the amount of disinfectant that may get into ponds and wetlands if field biologists or amateur naturalists disinfect their boots and other

materials. Additionally, the water volume of such amphibian breeding sites is much higher than the water volume of the experimental mesocosms. Hence, our treatments, even the low dose, represent worst case scenarios. If there are no effects of such a high dose, then there will be no effects at lower and more realistic doses.

Whereas harmful effects of bleach were obvious (figures 1 to 3), Virkon S had no detectable effects on tadpoles and zooplankton in our experiment. It could well be that Virkon S has effects on other traits that were not measured (e.g. damage of internal tissue). For example, Bernabo et al. (2008) reported that the pesticide endosulfan had effects on the ultrastructure of gills of tadpoles of *Bufo bufo*. Similarly, Virkon S may be harmful for other species or interactions with other stressors may make it harmful (Relyea, 2003). However, it should be noted that it is impossible to show that Virkon has no effects; experiments can only show that some factor has an effect on some response variable.

We believe that the endpoints that we selected for our study were well chosen (Bridges and Semlitsch, 2005). First, tadpoles of many pond-breeding anurans are more likely to be exposed to disinfectants against *Bd* than adults. Second, the behavioural trait we analysed is known to affect tadpole growth and ecological interactions (Werner and Anholt, 1993; Rist et al., 1997). Third, growth and survival can affect population dynamics (Altwegg and Reyer, 2003; Schmidt et al., 2005). Whether individual-level responses translate into population-level responses depends on density dependence and is an open question (Forbes and Calow, 2002; Schmidt, 2004). However, if there were harmful effects of Virkon S, the precautionary principle would suggest a prudent usage of potentially harmful chemicals.



Researchers and conservationists working on amphibians should disinfect their boots and materials to prevent further spread of *Bd* (NSW Parks and Wildlife Service, 2001; Australian Government Department of Environment and Heritage, 2006; Fisher and Garner, 2007; Gascon et al., 2007; Dejean et al., 2007; Young et al., 2007). Whenever there is a choice, the disinfectant that is least harmful should be used. Because the contamination of wetlands and ponds with bleach can have negative effects on amphibians and other aquatic organisms, it is necessary that bleach is used with great care. It must not come enter water bodies or come into contact with amphibians (for example, boots and materials could be rinsed with water after disinfection). It may be necessary to wash boots and other equipment with water after the use of disinfectants. In conclusion, because Virkon S appears to be a highly efficient disinfectant against *Bd* (Johnson et al., 2003) and had no measurable effects on the animals tested in this study, we recommend its use during field work.

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**Table 1.** Results of the general linear model analysis (with binomial errors) for tadpole survival. Terms were added sequentially (first to last).

Factor	d.f.	deviance	d.f.	Residual deviance	<i>P</i>
<i>Bufo bufo</i>					
Null			29	259.6	
Disinfectant	2	139.2	27	120.3	< 0.0001
Dose	1	72.6	26	47.6	< 0.0001
Interaction	2	9.2	24	38.3	0.0100
<i>Rana temporaria</i>					
Null			29	259.6	< 0.0001
Disinfectant	2	139.2	27	120.3	< 0.0001
Dose	1	72.6	26	47.6	0.0100
Interaction	2	9.2	24	38.3	

Table 2. Results of the linear model analysis (with normal errors) for tadpole mass.

Factor	d.f.	Mean squares	<i>F</i>	<i>P</i>
<i>Bufo bufo</i>				
Number of survivors	1	0.0047	5.03	0.0363
Treatment	3	0.0023	0.83	0.4923
Residual	20	0.0009		
<i>Rana temporaria</i>				
Number of survivors	1	0.0418	14.23	0.0011
Treatment	3	0.0110	3.76	0.0270
Residual	20			

**Fig. 1.** Effects of experimental treatments (controls pooled) on the behaviour of tadpoles of *Bufo bufo* and *Rana temporaria*. The response variable is the proportion feeding (where 100% is the number of tadpoles visible); this is an average across seven to ten measurements per experimental unit. “high” and “low” refer to the dose of the disinfectant. No tadpole survived in the high dose of bleach treatment. Each dot represents one mesocosm (i.e. experimental unit).

**Fig. 2.** Effects of experimental treatments (controls pooled) on survival and mass of *Bufo bufo* and *Rana temporaria* tadpoles. “high” and “low” refer to the dose of the disinfectant. No tadpole survived in the high dose of bleach treatment. Each dot represents one mesocosm (i.e. experimental unit).

**Fig. 3.** Effects of experimental treatments (controls pooled) on zooplankton abundance. Zooplankton abundance is the sum of daphnids, ostracods and copepods. “high” and “low” refer to the dose of the disinfectant. No zooplankton survived in the high dose of bleach treatment. Each dot represents one mesocosm (i.e. experimental unit).

Fig. 1

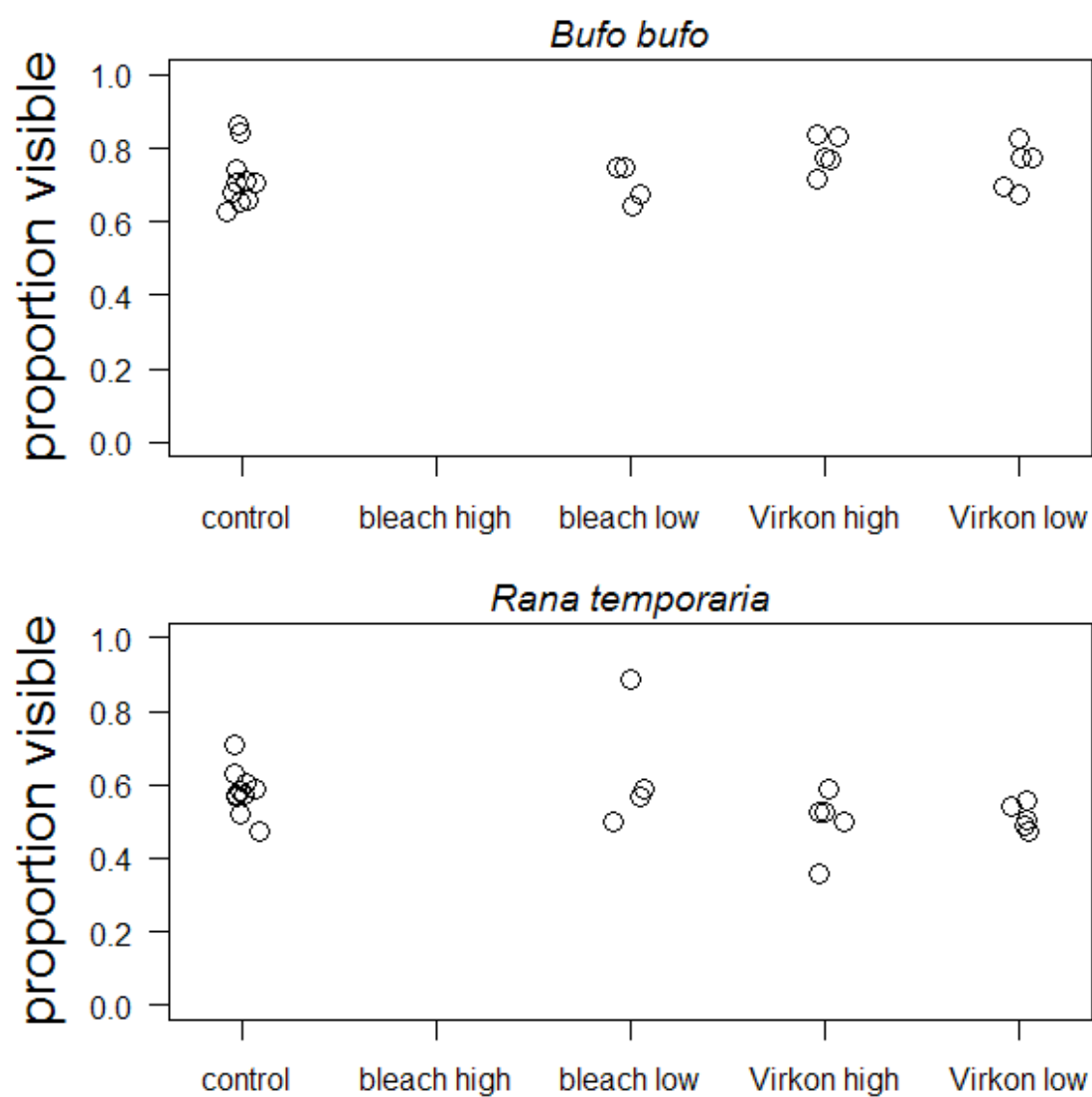


Fig. 2

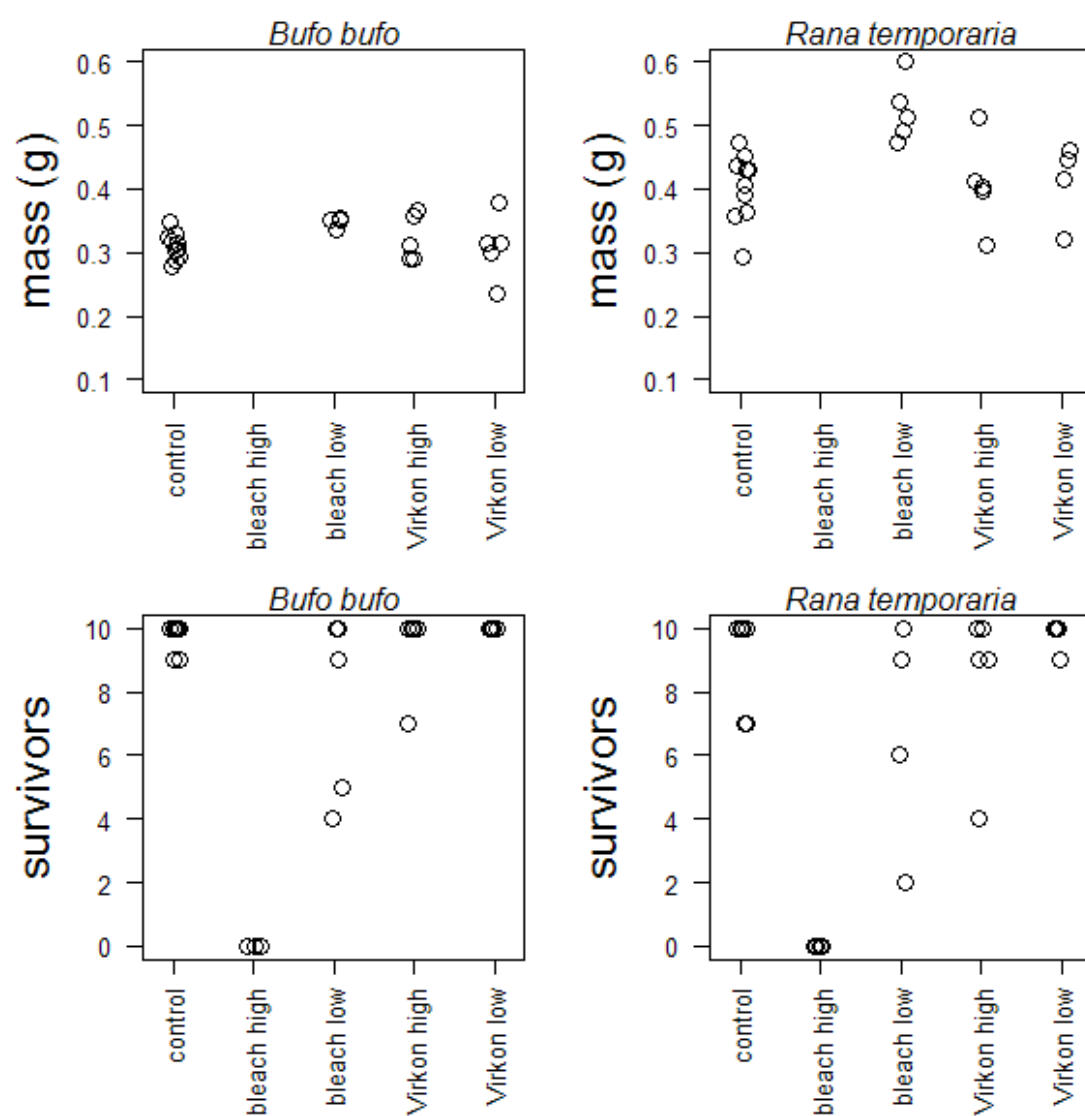




Fig. 3

